

AD-A179 928

DTIC FILE COPY

②

AD _____

Preparation and Characterization of Anti-Paralytic Shellfish Poison
Poly- and Monoclonal Antibodies

for

Development of Identification and Prophylaxis/Therapy Techniques

Annual/Final Report

Peter H Duquette, Richard A. Amos, Jerome C. Behrens,
Ross P. Chambers, Mark W. Josephson, and Patrick E. Guire

23 February 1987

Supported by
U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5202

Bio-Metric Systems, Inc.
9924 West Seventy Fourth Street
Eden Prairie, Minnesota 55344

DOD DISTRIBUTION STATEMENT

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official
Department of the Army position unless so designated by other
authorized documents.

DTIC
ELECTE
MAY 05 1987
E

20030121165

87 5 4 005

Unclassified
SECURITY CLASSIFICATION OF THIS PAGE

ADA 179 928

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT SECURITY CLASSIFICATION Unclassified		1b. RESTRICTIVE MARKINGS	
2. SECURITY CLASSIFICATION AUTHORITY		3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution unlimited	
4. DECLASSIFICATION / DOWNGRADING SCHEDULE			
PERFORMING ORGANIZATION REPORT NUMBER(S)		5. MONITORING ORGANIZATION REPORT NUMBER(S)	
NAME OF PERFORMING ORGANIZATION io-Metric Systems, Inc.		6b. OFFICE SYMBOL (If applicable)	
7a. NAME OF MONITORING ORGANIZATION			
ADDRESS (City, State, and ZIP Code) 924 West Seventy Fourth Street den Prairie, MN 55344		7b. ADDRESS (City, State, and ZIP Code)	
NAME OF FUNDING / SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command		8b. OFFICE SYMBOL (If applicable)	
9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-85-C-5202			
ADDRESS (City, State, and ZIP Code) ort Detrick, Frederick, MD 21701-5012		10. SOURCE OF FUNDING NUMBERS	
PROGRAM ELEMENT NO. 61102A		PROJECT NO. 1161. 102BS12	
TASK NO. AA		WORK UNIT ACCESSION NO. 103	
11. TITLE (Include Security Classification) reparation and Characterization of Anti-Paralytic Shellfish Poison Poly- and Monoclonal ntibodies for Development of Identification and Prophylaxis/Therapy Techniques (U)			
12. PERSONAL AUTHOR(S) eter H. Duquette, Richard A. Amos, Jerome C. Behrens, Ross C. Chambers, Mark W. Josephson,			
13. TYPE OF REPORT Annual / Final Report *		14. DATE OF REPORT (Year, Month, Day) 23 February 1987	
15. PAGE COUNT 20			
SUPPLEMENTARY NOTATION Annual for the period 16 Jul 86 - 31 Dec 86, Final for the period 15 Jul 85 - 31 Dec 86,			
COSATI CODES		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP	SUB-GROUP	
6	20		
6	01		
ABSTRACT (Continue on reverse if necessary and identify by block number)			
Significant progress was made toward synthesizing modified C-5 and C-6 guanidinium analogs as possible immunogens for use in producing polyclonal antibodies to the PSP. The C-5 guanidinium acid analogs were coupled to BSA or modified BSA. Rabbits were immunized with these analogs and the serum was examined for the possible production of useful antibodies to saxitoxin and the other PSP.			
DISTRIBUTION / AVAILABILITY OF ABSTRACT <input checked="" type="checkbox"/> UNCLASSIFIED / UNLIMITED <input type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS		21. ABSTRACT SECURITY CLASSIFICATION Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL cs. Judy Pawlus		22b. TELEPHONE (Include Area Code) 301/663-7325	
		22c. OFFICE SYMBOL SGRD-RMI-S	

Form 1473, JUN 86

Previous editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

Unclassified

Summary

→ The development of polyclonal antibodies having good cross-reactivity against the entire family of the paralytic shellfish poisons has been the focus of this research effort. One approach to this problem is the design of small hapten molecules which express the epitopes common to all the PSP toxins. Since the guanidinium ring systems are integral parts of all of the toxin molecules, synthetic routes to both the five- and the six-membered ring systems have been developed. In both cases, a carboxylic acid group is attached to the ring system for coupling to carrier protein via an activated ester route. The coupling of the five-membered guanidinium ring to a variety of carrier proteins and the antibody response to these conjugates in rabbits has been investigated.

*Keywords: toxins and antitoxins; saxitoxin;
antibodies; immunogens*

Accession For	
NTIS GRA&I	<input checked="checked" type="checkbox"/>
DTIC TAB	<input type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification	
By _____	
Distribution/	
Availability Codes	
Avail and/or	
Dist	Special
A-1	



Foreword

In conducting the research described in the report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" of the Institute of Laboratory Animal Resources, National Research Council.

(DHEW Publication No. (NIH) 78-23, Revised 1978).

Table of Contents

	<u>Page</u>
Summary	3
Foreword	4
Table of Contents	5
Identification and Significance of the Problem	6
Background	6
Rationale	7
Results	7
Discussion	12
References	13
Figure 1, The PSP	15
Figure 2, PSP Cross-Reaction Studies	16
Figure 3, Saxitoxin Potency Domains	17
Figure 4, C-5 Synthesis	18
Figure 5, C-6 Synthesis	19
Distribution List	20

Identification and Significance of the Problem

Massive dinoflagellate blooms in coastal waters are the cause of the phenomenon known as the "red tide" [1]. These organisms produce a very potent group of tetrahydropurine-based toxins involved in paralytic shellfish poisoning (PSP) ([2], Figure 1). The mode of action of the PSP is based upon their ability to act as potent blocking agents of the voltage-sensitive sodium ion channels in neuromuscular membranes [3-5]. They disrupt the ion passage which prevents signal transmission in the neuron; this effect can cause paralysis and death. Therefore, this group of compounds can serve as a source of significant potential risk to humans through accidental ingestion of shellfish which bioaccumulate the PSP (i.e., mussels, clams) or by the use of the PSP as chemical warfare agents.

Background

In the past, a variety of assays have been utilized for the assessment of PSP, but none of the tests are rapid enough for routine testing in the laboratory or field. The most widely utilized laboratory tests have been bioassays in animals with the current test for shellfish toxins being based upon a time-of-death mouse assay [6]. The advantage of the assay is that it indicates total toxicity; but has the following distinct disadvantages: 1) need to maintain mouse colony with mice in the 19-22 gram weight range, 2) sensitivity dependent on mouse strain, 3) possibility for underestimating marginally toxic clams, 4) subsequent time of death determination, 5) laborious to perform, and 6) relatively expensive [7]. Consequently, a variety of assay methods have been developed as alternatives to the mouse assay. These include the following: a) fluorometric and colorimetric methods based upon hydrogen peroxide oxidation [8-11] and b) high performance liquid chromatography methods [12-14]. Each of these assays has a serious disadvantage which has prevented it from replacing the mouse assay. The peroxide oxidation methods require following a strict protocol [9] and the neosaxitoxin sub-group of the PSP is not efficiently detected [15]. The HPLC methods require extensive sample preparation and the need for expensive equipment and trained personnel. Furthermore, the HPLC methods for detection of the PSP toxins require the use of post-column derivatization since they do not give a significant UV/VIS absorption band.

An attractive alternative to the above methods is the development of a PSP assay based on the selectivity and high affinity characteristics of PSP binding proteins. Presumably an assay could be based on the PSP binding proteins from excitable nerve membranes [16]. Although the binding characteristics of these proteins would probably be excellent (high K_D [17], good PSP cross-reactivity [based on PSP toxicities]), their isolation in sufficient quantity for application in a routine assay would be expected to be difficult [3]. At least one soluble binding protein, which would alleviate the isolation problem, has been identified but the quantity available is extremely limited [18,19].

In contrast to the binding proteins which are available only in limited quantities, antibodies can be produced in much larger amounts. Several previous attempts have been made to prepare anti-PSP antibodies [20,21]. Koehn *et. al.* were unable to prepare immunogenic PSP-protein conjugates [20] and the antisera prepared by Johnson *et. al.*, which was raised in rabbits using a labile PSP-protein conjugate, gave relatively poor anti-PSP antisera [21]. The sensitivity of the hemagglutination assay which was based on these antisera was marginal (ca. 1 nmole/ml minimum detection level) [22]. Recently, Chu and co-

workers have obtained more impressive but still limited results using Johnson's immunogen in combination with a enzyme immunosorbent assay format [23]. Our studies have led to successful production and characterization of an anti-PSP antisera based on a stable saxitoxin (STX) derivative-bovine serum albumin (BSA) conjugate [24]. This antisera has been used in the development of an STX radioimmunoassay [24] and has been evaluated for use in anti-STX therapy [25].

Rationale

We have demonstrated that it is possible to produce an anti-STXol antisera which will function in development of therapeutic and analytical protocols for STX and closely related PSP [24]. However, the demonstration preparation has significant drawbacks in that it is not cross-reactive against the NEO subgroup of the PSP and because it is only a weak competitor with the PSP binding protein (Figure 1).

The goals of this project are to produce anti-PSP antibodies which will be useful for both the analysis of the PSP and for prophylaxis/therapy of PSP poisoning. Therapeutic use of the antibody will require high PSP affinity so that the antibody can compete with the PSP binding protein for the toxin. Analytical use of the antibody will require high cross-reactivity so that the antibody and the assay will be readily usable for the determination of total PSP (Figures 2,3).

Generally, these goals are somewhat exclusive because the ability to recognize haptens which have significant structural variations requires that the antibody be "flexible" enough in its binding to allow access by differing functional groups. It is possible that a single functional group (epitope), which is consistently retained throughout the PSP series, will have a high binding affinity.

Analysis of the PSP (Figure 1) and the results of the cross-reactivity studies (Figure 2) indicated that the most useful binding site for an anti-PSP antibody might be based upon an immunogen which contains only the five-membered N-7,8,9 guanidinium ring. In order to provide a point for covalent attachment to protein, the ring system was further substituted with a carboxylic acid. Much of the synthetic work was focused upon the synthesis of this guanidinium acid (compound 3, Figure 4), referred to as the C-5 hapten. Because of the closely related synthetic chemistry, the analogous six-membered ring (compound 6, Figure 5, C-6 hapten) was also prepared using methods developed for the C-5 compound. These analogs would exhibit good cross-reactivity with antibody and thus antibodies produced against the C-5 and C-6 guanidinium analogs would be extremely useful in the development of an enzyme immunoassay for detecting total PSP content.

Results

Chemistry

C-5 Guanidinium Acid

The C-5 hapten was prepared by the scheme outlined in Figure 4. S,S-Dimethyl-N-tosyliminodithiocarbonimidate, 1, was prepared by the reaction of p-toluenesulfonamide with carbon disulfide in a concentrated aqueous NaOH solution with a DMF co-solvent. The resulting anion was then trapped by alkylation with methyl iodide, giving a 77.6% yield of 1 after recrystallization from methanol. The melting point (106-107°C) was in close

agreement with the literature value (109°C) [26].

The tosylate protected guanidinium acid, 2, was prepared by reaction of 1 with commercially available 2,3-diaminopropionic acid monohydrochloride. The diamino acid was first neutralized with two equivalents of aqueous NaOH and the resulting salt solution was refluxed with 1 in an aqueous ethanol solvent for 24 hours. The resulting product was recrystallized from water yielding a powdery white solid (84% yield, m.p. 208-210°C with decomposition) whose structure was confirmed by IR and NMR analysis.

The detosylation of the tosylate protected guanidinium acid 2 proved to be a very difficult step. The literature has reported that such detosylations can be accomplished using phenol in refluxing HBr [27,28]. When 2 was refluxed for two hours with this reagent combination, thin layer chromatography confirmed the consumption of starting material and the formation of the desired guanidinium product. However, numerous byproducts made purification of the product very difficult. After extensive experimentation, a workup procedure was established which consisted of an initial removal of the HBr solvent under reduced pressure. The resulting oil was then diluted with water and the organic soluble impurities were removed by successive extractions with diethyl ether and ethyl acetate. The remaining water soluble residue was then subjected to a cationic ion-exchange column (AG 50W-X4, Bio-Rad) for final purification. The column retained the charged guanidinium salt, permitting the removal of water soluble, uncharged impurities as well as the substitution of chloride for bromide using elution with HCl. Final isolation of 3 gave an 88% yield with structure confirmation by IR and NMR.

In order to quantitate the coupling efficiency of the C-5 hapten and to monitor antibody response to the C-5 immunogen, the radiolabeled analog of 3 has been synthesized using the same procedure as described above. The starting 2,3-diaminopropionic acid-3-¹⁴C was prepared for us (Moravek Biochemical, Inc., Brea, Calif.) and was reacted with S,S-dimethyl-N-tosyliminodithiocarbonimide to form the tosylated guanidinium acid 2 with the ¹⁴C label in the ring system. The formation of labeled 2 was not a clean reaction and required extensive purification by preparative HPLC (polar aminocyanophase column; Whatman) to remove numerous byproducts resulting from minor impurities in the diamino acid. The detosylation of 2 with HBr and phenol, followed by the workup as described above proceeded quite well to give 3 as confirmed by NMR analysis. Preliminary data showed a specific activity of approximately 20 mCi/mmole.

C-6 Guanidinium Acid

In addition to the C-5 hapten molecule, we have also been involved in the synthesis of the corresponding C-6 guanidinium acid (6, Figure 5). This molecule could also be used in the preparation of a useful immunogen for producing antisera which would have a high binding affinity for all of the PSP's. Since it is apparent that alterations of various portions of the PSP molecule can have a significant effect on potency (Figure 3), it may be beneficial to produce alternative immunogens which would possess structural features similar to those observed in portion II of the saxitoxin molecule (Figure 3).

The C-6 guanidinium acid was prepared by procedures similar to the C-5 hapten. Commercially available (S)-(+)-2,4-diaminobutyric acid dihydrochloride was first neutralized with three equivalents of aqueous NaOH and then refluxed 22 hours with 1 in an aqueous ethanol solvent. The tosylate protected guanidinium acid 5 was purified by recrystallization from water, giving an 85% yield of a white powdery solid, melting point 216-220°C (lit. 202-203°C) [29].

The structure was confirmed by IR and NMR analysis.

The detosylation of 5 was accomplished using the HBr and phenol reflux procedure outlined previously for the C-5 hapten. The crude product was purified as before using the selective extraction of organic soluble impurities with diethyl ether and ethyl acetate, followed by ion-exchange purification of the water soluble product. A 93% yield of a pale yellow solid was achieved, which upon recrystallization from isopropanol-ether gave a 54% yield of white crystals, m.p. 209-212°C (lit. m.p. 210-211°C) [29].

Guanidinium Alcohol Synthesis

Compounds 3 and 6 rely upon the carboxylic acid group for coupling to protein via stable amide linkages, formed by reaction of activated esters with free amine groups on the protein. An alternative approach considered was to use the corresponding guanidinium alcohols obtained by reduction of the carboxylic acid group. The alcohol could be coupled using 1,1'-carbonyldiimidazole (CDI) by formation of stable carbamate linkages with amine groups on the protein. Our earlier success in coupling saxitoxinol to BSA using CDI provided strong precedent for this approach [24].

The carboxylic acid group in 2 is easily reduced by treatment with lithium aluminum hydride (LAH) in THF for one hour at 25°C and then 30 minutes at reflux. The excess LAH is quenched by the addition of an equal volume of ethyl acetate, followed by washing with water and a back extraction with ethyl acetate to give the tosylate protected C-5 guanidinium alcohol 4. The yield of a viscous yellow oil was 89%.

In a similar fashion, the tosylate protected C-6 guanidinium alcohol 7 was prepared by LAH reduction of 5, providing an 85% yield of a white crystalline product, m.p. 170-173°C. Structural confirmation was provided by IR and NMR analysis.

Before coupling the alcohol to protein, removal of the tosylate protecting group to give 8 was required. At the time of the termination of this contract, only preliminary work had been completed on the synthesis of 8 and no work had been started on the corresponding deprotected C-5 guanidinium alcohol. However, the information obtained on 8 was very encouraging. Reduction of 6 with LAH in THF and the deprotection of 7 with HBr and phenol led to products which were chromatographically identical. The fact that two completely different routes led to the same product would appear to confirm the identity of 8. Termination of the contract prevented complete characterization of the products.

Immunogen Preparation

In addition to the preceding synthesis work, a significant amount of effort was directed toward producing suitable immunogens by coupling the C-5 guanidinium acid to protein. The approaches initially considered for immunogen preparation were: 1) coupling of the N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) activated C-5 ester directly to bovine serum albumin (BSA); 2) coupling of the activated ester derived from the N-hydroxysuccinimide (NHS) or N-hydroxysulfosuccinimide (sulfonHS) to the protein; 3) preparation of C-5 guanidinium-4-aminobutyric acid (GABA) or 3,3'-diaminodipropylamine (DADPA) analogs and coupling of these spacer groups to BSA by either of the above activation methods; and 4) direct coupling of the C-5 guanidinium acid to protein by the use of 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (EDC).

All of the above approaches were explored with varying success in terms of

immunogen preparation. The direct coupling of 3 using EDC failed to give any indication of the desired immunogen. Examination of the reaction product indicated that only intramolecular crosslinking of the protein had been produced. The preparation of C-5 haptens having GABA and DADPA spacer groups on the carboxylic acid group of 3 proved very difficult. However, small quantities of both conjugates were obtained and purified by the use of silica gel GF preparative plates. Attempts were made to couple the GABA analog to BSA using the EEDQ activated ester and the sulfonOS activated ester or to directly couple the DADPA analog to BSA using water soluble EDC. Neither of these analogs could be coupled to BSA in sufficient yield to be useful as immunogens.

We also explored the option of attaching a long spacer chain terminated in a free hydroxyl group to the C-5 guanidinium acid. The free base of the C-5 hapten was prepared by ion-exchange on Amberlite IRA-400 and it was then successfully alkylated with 11-bromo-1-undecanol. However, attempts to couple this adduct to BSA by CDI activation gave very poor results with only 1-2 moles of hapten per mole of BSA. The use of 3-bromo-1-propanol as the alkylating agent failed to give any of the desired alkylation product and thus no immunogen preparation could be attempted.

Our first apparent success in immunogen preparation came by use of a sulfonOS activated ester of the C-5 guanidinium acid in reaction with BSA. The free base of the C-5 hapten was prepared by passing the HCl or HBr salt of the hapten (in ethanol) through an Amberlite IRA-400 (OH-form) ion-exchange column. The C-5 carboxyl free base was converted to the activated sulfonOS ester by reaction with sulfonHS in DMF using dicyclohexylcarbodiimide (DCC) as a dehydrating agent. After stirring six hours at room temperature, the reaction was transferred to a 4°C cold room for another 16 hours. The resulting dicyclohexylurea (DCU) was removed by filtration of the cold solution and the sulfonOS ester (in 150 molar excess) was mixed with BSA or modified BSA in 0.2 M NaHCO₃, pH 9. The reaction mixture was stirred at room temperature for 90 minutes and then was transferred to a 4°C cold room for an additional 16 hours. The resulting immunogen was then dialyzed extensively against PBS, concentrated by ultrafiltration and used for the immunization of rabbits (or stored at -20°C).

The use of this immunogen for polyclonal antibody production failed to produce any antisera against the guanidinium acid as determined by use of the radiolabeled material. Our preliminary data based on amine analysis of the protein had indicated a loss of amines corresponding to an incorporation of 10-20 moles hapten/mole of protein. However, the lack of immunological response leads us to believe that no coupling of the guanidinium acid occurred and that the consumption of amines is attributable to acetate present in the salt of the guanidinium product. Furthermore, it would be predicted that the incorporation of the guanidine group should not lead to a loss of amines on the protein, but instead should increase the available amine content.

Because of the difficulties encountered in achieving effective coupling of the C-5 guanidinium acid to protein, we decided to pursue modifications of the carrier protein prior to coupling of the hapten molecule. Toward this goal, three modified BSA preparations were produced: 1) direct coupling of DADPA to the carboxylic acid moieties on BSA using EDC activation, thus increasing the effective amine content; 2) acetic anhydride addition to BSA to acetylate the free amines on BSA, followed by DADPA coupling to the remaining carboxylic acid groups using EDC activation; and 3) addition of succinic anhydride to BSA with subsequent coupling of DADPA to all carboxylic acid groups to give an increased number of amine sites available for coupling to the activated ester of 3.

The following procedures describe the preparation of the above mentioned

modified proteins. If the protein (BSA) is first acetylated or succinylated, the BSA (300 mg) is dissolved in 0.2 M NaHCO_3 (pH 8.0-8.5) and the appropriate anhydride is added slowly (over two hours) while keeping the pH between 8.0 and 8.5. After addition of the anhydride, the pH is adjusted to 8.0 using 1 N NaOH and the mixture is stirred 30 minutes at room temperature. The mixture is then dialyzed at 4°C against 0.1 M morpholine ethanesulfonic acid (MES), pH 6.0. To add DADPA spacers to the pretreated or native BSA, 1.0 ml of DADPA is added to 2.0 ml of water containing 70 mg of MES. The pH is carefully adjusted to 5.5 using 12 N HCl and the BSA and DADPA solutions are mixed together. The EDC (4 x 200 mg) is then added at 30 minute intervals while maintaining the mixture at room temperature. Following the last addition of EDC, the solution is transferred to a 4°C cold room and stirred 16-18 hours. The mixture is then dialyzed extensively against PBS and concentrated by ultrafiltration to a level of 60-70 mg protein/ml solution. Amine analysis of the products gave results consistent with the various treatments and this procedure will serve as the model system for modification of other carrier proteins (i.e., bovine gamma globulin, sheep gamma globulin).

We were successful in coupling the C-5 guanidinium acid to all three modified carrier proteins as indicated by shifts in the UV spectrum of the protein and increased amine values of the protein. Each of these immunogens was then used to immunize rabbits using the protocol described in a following section. Examination of the sera from all of the rabbits for antibody production up to 16 weeks using an Ouchterlony assay indicated that anti-C-5 antisera was being produced. However, examination of binding of radiolabeled saxitoxinol and C-5 hapten failed to indicate any evidence of binding of the labeled molecules.

The difficulties encountered in achieving effective coupling of the C-5 hapten molecule to protein led us to recognize that the potential exists for the guanidine group to react with the NOS activated ester. The guanidine group, in the free base form, is a very basic moiety and is potentially a very good nucleophile for reaction with the activated ester to give a polymer of the C-5 hapten. Indeed, in early experiments, designed to allow isolation of the NOS ester, the disappearance of 3 could be monitored by chromatographic means but no evidence of the activated ester could be found. The use of excess DCC in these experiments may have consumed the HCl present as the guanidinium hydrochloride salt, leaving the free base to react. In experiments conducted just prior to the termination of the contract, it was found that limited amounts of DCC permitted chromatographic observation of the NOS ester. Furthermore, this product was reacted with a model primary amine, p-nitrophenethylamine, to give a new amide adduct, thus demonstrating the viability of the NOS ester as an acylating agent. It seems quite probable that this improved preparation of the activated esters could lead to substantial improvements in protein loading.

Polyclonal Antibody Production

Six rabbits for each of the immunogens prepared were injected intracutaneously in multiple sites (40 sites - 0.1 mg/rabbit) along the back with an emulsion containing a 50% mixture of complete and incomplete Freund's Adjuvant. The rabbits were given subcutaneous booster injections (0.2 mg/rabbit in incomplete Freund's Adjuvant) at three week intervals. Examination of the sera began one week following the first booster injection.

Discussion:

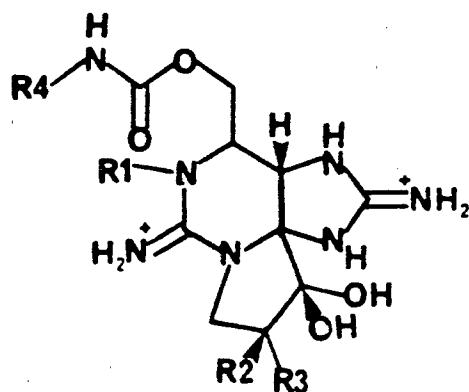
The generation of a single antibody to the entire class of PSP is made difficult by the significant differences in structures throughout the series (Figure 1). One approach to this problem is to focus on the portion of the molecule which is common to all members of the family, utilizing that structure fragment as a hapten for antibody production. In this manner, the opportunity exists for antibody production which would exhibit good cross-reactivity toward all the PSP. In keeping with this approach, the recent focus of our research has been on the use of the five-membered guanidinium ring species (Figure 2) as the hapten of choice. The use of the five-membered guanidinium ring coupled to carrier proteins failed to illicit any antibody production in rabbits which would bind the radiolabeled guanidinium or cross-react with any of the PSP. This lack of immunological response may be due to any one of the following factors: a) no significant production of appropriate immunogens formed by coupling the guanidinium species to carrier protein due to a competitive intermolecular reaction of the guanidinium NOS ester and guanidinium free base, b) the guanidinium species is too closely related to naturally occurring purine bases and thus will not elicit an immunological response, c) not the correct choice of animal species (rabbit) for antisera production, and d) poor choice of carrier protein. Despite this unsuccessful approach to producing antibodies which possessed a high degree of cross-reactivity to all the PSP, much useful information was obtained concerning the methods for covalent coupling of saxitoxin or analogs (guanidinium analog) to proteins for the development of a stable enzyme-hapten conjugate. This information will be useful in the development of stable enzyme-hapten conjugates necessary for the successful preparation of excellent enzyme immunoassays for saxitoxin and/or total PSP content.

References

1. Boyer, G.L., C.F. Wichmann, J. Mosser, E.J. Schantz, and H.R. Schnoes in Toxic Dinoflagellate Blooms, D.L. Taylor and H.H. Seliger (Eds), Elsevier North Holland, Inc., New York, NY, pp. 373-376 (1979).
2. Hall, S. and P.B. Reichardt, "Cryptic Paralytic Shellfish Toxins," in E.P. Ragelis, Ed. Seafood Toxins, ACS Symposium Series 262, Washington, DC, pg. 113 (1984).
3. Agnew, W.S., S.R. Levinson, J.S. Brabson, and M.A. Raftery, Proc. Natl. Acad. Sci. USA 75: 2606 (1978).
4. Catterall, W.A., Annu. Rev. Pharmacol. Toxicol. 20: 15 (1980).
5. Kao, C.Y., Fed. Proc. 40: 30 (1981).
6. Association of Official Analytical Chemists in: Official Methods of Analysis, 14th Edition, Washington, DC, page 344 (1984).
7. Shimizu, Y. and E. Ragelis in Toxic Dinoflagellate Blooms, D.L. Taylor and H.H. Seliger (Eds), Elsevier North Holland, Inc., New York, NY, pp 453-455 (1979).
8. Bates, H.A., H. Rapoport, "A Chemical Assay for Saxitoxin, the Paralytic Shellfish Poison," J. Agric. Food Chem. 23: 237 (1975).
9. Bates, H.A., R. Kostriken, and H. Rapoport, "A Chemical Assay for Saxitoxin. Improvements and Modifications," J. Agric. Food Chem. 26: 252 (1978).
10. Gershey, R.M., R.A. Neve, D.L. Musgrave, and P.B. Reichardt, "A Colorimetric Method for Determination of Saxitoxin," J. Fish Res. Board Can. 34: 559 (1977).
11. McFarren, E.F., E.J. Schantz, J.E. Campbell, and K.H. Lewis, "Chemical Determination of Paralytic Shellfish Poison in Clams," J. Assoc. Off. Agric. Chem. 41: 168 (1958).
12. Robinson, K.A., Biochem. Biophys. Acta 687: 315 (1982).
13. Sullivan, J.J. and W.T. Iwaoka, J. Assoc. Off. Anal. Chem. 66: 297 (1982).
14. Sullivan, J.J., M.M. Wekell, and L.L. Kentala, J. Food Sci. 50: 26 (1985).
15. Bose, R.J. and J.E. Reid in Toxic Dinoflagellate Blooms, D.L. Taylor and H.H. Seliger (Eds), Elsevier North Holland, Inc., New York, NY, pg. 399-402 (1979).
16. Weigle, J.B. and R.L. Barchi, FEBS Letters 54: 813 (1974).
17. Narashi, T., Physiol. Rev. 54: 813 (1974).
18. Doyle, D.D., M. Wong, J. Tanaka, and L. Barr, Science 215: 1118 (1982).

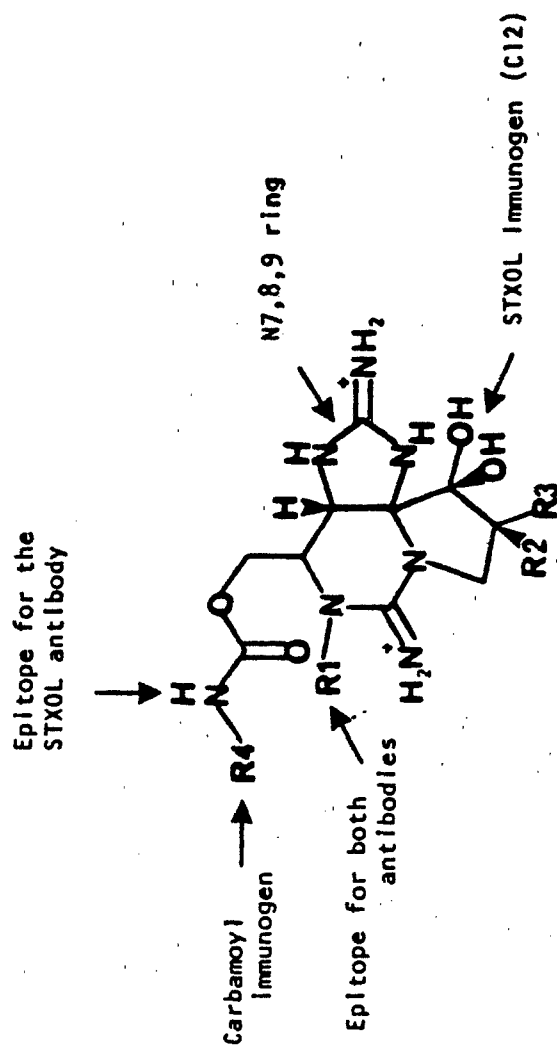
19. Hartshorne, R.P. and W.A. Catterall, Proc. Natl. Acad. Sci. USA **78**: 4620 (1981).
20. Koehn, F.E., V.E. Ghazarossian, E.J. Schantz, H.K. Schnoes, and F.M. Strong, Bioorg. Chem. **10**: 412 (1981).
21. Johnson, H.M., P.A. Frey, R. Angelotti, J.E. Campbell, and K.H. Lewis, Proc. Soc. Exp. Biol. Med. **117**: 425 (1964).
22. Johnson, H.M. and G. Mulberry, Nature **211**: 747 (1966).
23. Chu, F.S. and T.S.L. Fan, J. Assoc. Off. Anal. Chem. **68**: 13 (1985).
24. Carlson, R.E., M.L. Lever, B.W. Lee, and P.E. Guire, "Development of Immunoassays for Paralytic Shellfish Poisoning: A Radioimmunoassay for Saxitoxin," in E.P. Ragelis, Ed. Seafood Toxins, ACS Symposium Series **262**: Washington, DC, p. 181 (1984).
25. Davio, S.R., "Neutralization of Saxitoxin by Anti-Saxitoxin Rabbit Serum," Toxicon **23**: 669 (1985).
26. Gompper, R.P. and W. Hagele, "N-Sulfonyl-iminodithiokohlensaureester und N-Sulfonyl-dithiourethane," Chem. Ber. **99**: 2885 (1966).
27. Snyder, H.R. and R.E. Heckert, "A Method for the Rapid Cleavage of Sulfonamides," J. Am. Chem. Soc. **74**: 2006 (1952).
28. Snyder, H.R. and H.C. Geller, "Cleavage of Sulfonamides with Aqueous Hydrobromic Acid and Phenol. II," J. Am. Chem. Soc. **74**: 4864 (1952).
29. Rodricks, J.V. and H. Rapoport, "Synthesis of Cyclic Guanidines," J. Org. Chem. **36**: 46 (1971).

Figure 1. The PSP



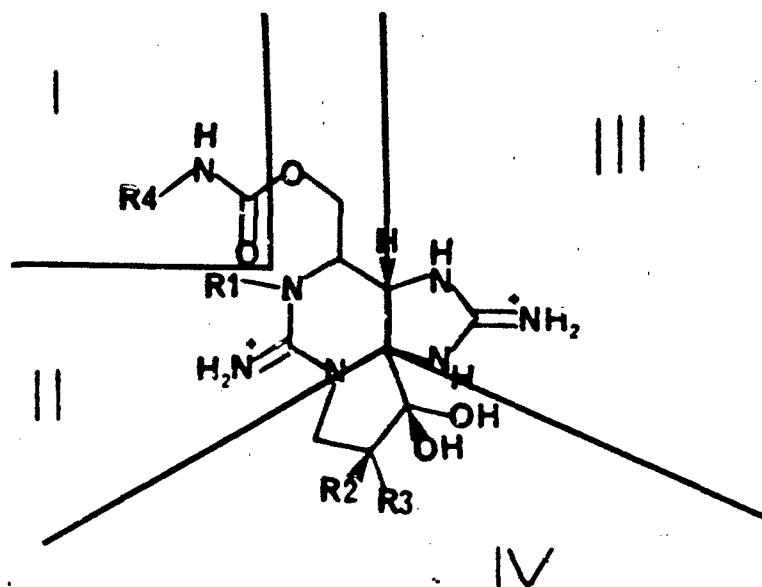
<u>Compound</u>	<u>R1</u>	<u>R2</u>	<u>R3</u>	<u>R4</u>
IA-- Saxitoxin (STX)	H	H	H	H
IB - Gonyautoxin2 (GTX2)	H	H	OSO ₃	H
IC - Gonyautoxin3 (GTX3)	H	OSO ₃	H	H
ID - Neosaxitoxin (NEO)	OH	H	H	H
IE - Gonyautoxin1 (GTX1)	OH	H	OSO ₃	H
IF - Gonyautoxin4 (GTX4)	OH	OSO ₃	H	H
IG - Saxitoxinol (STXOL)	H	H	H	H

Figure 2. PSP Cross-Reaction Studies



**** STX --- R1,2,3,4 = H
 NEO --- R1 = OH
 R2,3,4 = H

Figure 3. SAXITOXIN POTENCY DOMAINS



SAXITOXIN

(R1,R2,R3,R4 = H)

- I Potency reduced 20-100% on decarbamylation or sulfation
- II Potency increased up to 4 fold on addition of N1 -OH
- III Considered important for binding
- IV Potency reduction 0-90% on reduction of the carbonyl or sulfation

Figure 4
C-5 Synthesis

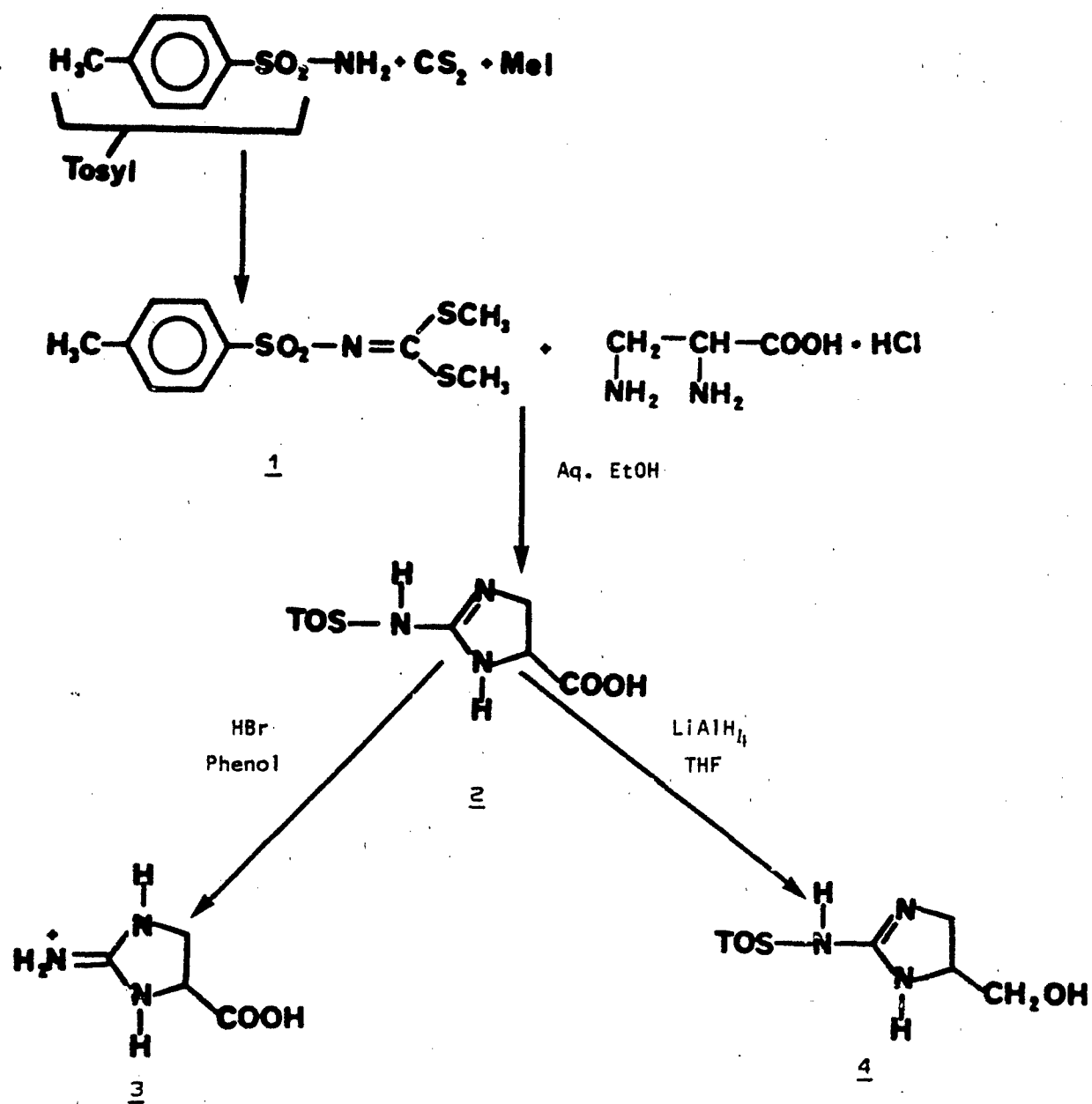
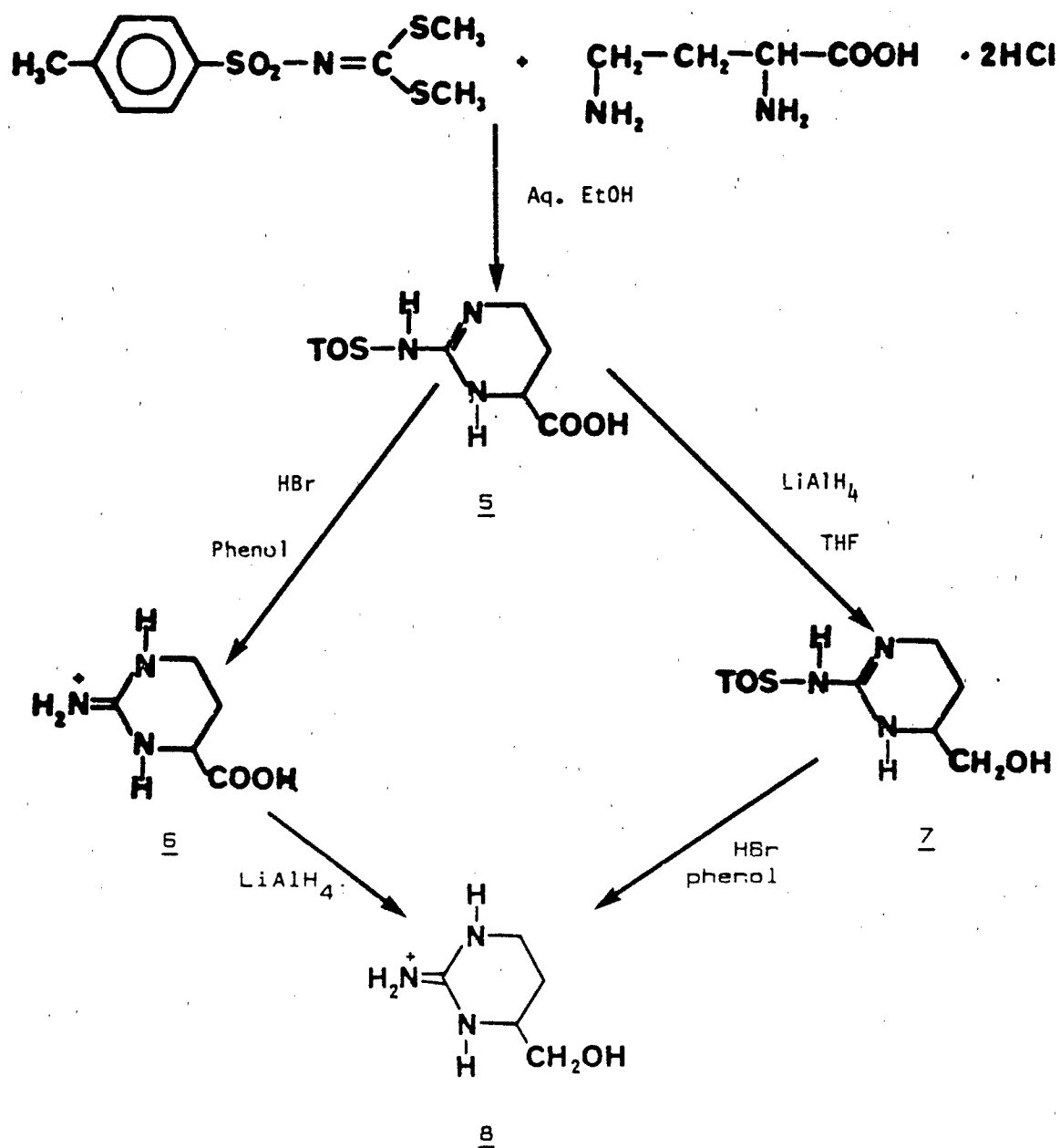


Figure 5
C-6 Synthesis



DISTRIBUTION LIST

5 copies

Commander
U.S. Army Medical Research Institute of
Infectious Diseases
ATTN: SGRD-UIZ-M
Fort Detrick, Frederick, MD 21701-5011

1 copy

Commander
U.S. Army Medical Research and Development Command
ATTN: SGRD-RMI-S
Fort Detrick, Frederick, MD 21701-5012

2 copies

Defense Technical Information Center (DTIC)
ATTN: DTIC-DDAC
Cameron Station
Alexandria, VA 22304-6145

1 copy

Dean
School of Medicine
Uniformed Services University of the
Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814-4799

1 copy

Commandant
Academy of Health Sciences, U.S. Army
ATTN: AHS-CDM
Fort Sam Houston, TX 78234-6100